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PREPARATION OF 5'-GUANYLIC ACID
[5'-GUANIRUSAN NO SEIZOHO]

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TITLE (54): METHOD FOR MANUFACTURING 5'-GUANYLIC ACID

FOREIGN TITLE [54A]: 5'-GUANIRUSAN NO SEIZOHO

Specifications /547*

1. Title of the Invention

Method for Manufacturing 5'-Guanylic Acid

2. Claim(s)

(1) A method for manufacturing 5'-guanylic acid characterized by catalyzing items (a) and (b) below in a water-based medium containing item (c) and collecting 5'-guanylic acid from said medium.

(a) cultured product or bacterial cells of Escherichia coli or treated products thereof having the ability to produce 5'-guanylic acid from 5'-xanthylic acid and ammonia and/or L-glutamine in the presence of adenosine triphosphate and having the ability to convert adenosine phosphate into adenosine triphosphate by using an energy donor other than a phosphorus oxide

(b) 5'-xanthylic acid, ammonia and/or L-glutamine

(c) energy donor other than a phosphorus oxide

(2) The method of Claim 1 wherein a transformed strain obtained by transforming Escherichia coli using a DNA fragment containing guanylic acid synthase, vector DNA, and recombinant DNA is used.

(3) The method of Claim 1 or 2 wherein a surfactant and/or organic solvent is present in the water-based medium.

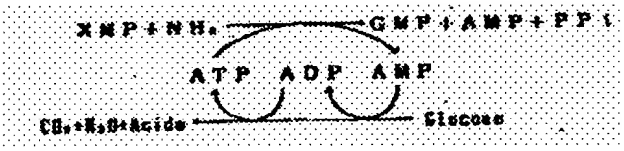
3. Detailed Specifications

The present invention relates to a method for manufacturing 5'-guanylic acid (abbreviated "GMP," hereafter), and in further detail, a method for manufacturing GMP by using Escherichia coli having the ability to produce

*Number in the margin indicates pagination in the foreign text.

GMP from 5'-xanthylic acid (abbreviated "XMP," hereafter), ammonia and/or L-glutamine and having the ability to convert adenosine phosphate (abbreviated "XMP," hereafter) to adenosine triphosphate (abbreviated "ATP," hereafter) by utilizing an energy donor other than a phosphorus oxide, and a method for manufacturing GMP in the above-mentioned method in which bacteria containing a vector in which a guanylic acid synthase gene (may be abbreviated "guaA," hereafter) region is used.

GMP is heavily demanded as a seasoning, and development of an inexpensive method for manufacturing it is desired. So far, (1) a method for manufacturing it by decomposing ribonucleic acid extracted from yeast fungal cells, (2) a method for chemically phosphorylating guanosine produced in a fermentation method, (3) a method for converting XMP produced in a fermentation method to GMP by using bacteria belonging to brevibacteria or coynebacteria (Tokuho No. 46-39069 and Tokugan 57-187050), among other methods are known. Method (3) is an excellent method in which ATP, /548 which is a high-energy compound required for transformation, as shown by the formula below is reproduced from AMP by digesting an inexpensive energy donor (glucose, or the like).



Furthermore, depending on the drug tolerance, a method in which a mutant, which reinforces the activity of a converting enzyme (guanylic acid synthase; alias: xanthylic acid aminase, abbreviated "GMP synthase," hereafter) from XMP to GMP is used, (Biotechnol. Bioengineer., 13 (1971):229-240, among other methods also is used.

In recent years, it has been relatively easy to reinforce a specific enzyme activity on Escherichia coli by applying recombinant gene technology.

As a result of performing various studies based on such a background, the inventors of the present invention discovered that GMP could be manufactured in the presence of phosphate ions and magnesium ions using E.coli having the ability to produce GMP from XMP and ammonia and/or L-glutamine and having the ability to convert AMP to ATP by utilizing an energy donor other than a phosphorus oxide and that GMP could be more efficiently manufactured using Escherichia coli having the above-mentioned properties for performing a transformation using a DNA fragment containing a guanylic acid synthase gene of an Escherichia coli chromosome, vector DNA and recombinant DNA, which led them to completing the present invention.

The present invention will now be described in detail.

Any Escherichia coli having the ability to produce GMP from XMP, ammonia and/or L-glutamine and an energy donor other than a phosphorus oxide usable by Escherichia coli can be used for the Escherichia coli (E. coli) used in the present invention. Ideally, E. coli PL1068 (see J. Gen. Microbiol., 123 (1981):27-37 (1981), E. coli KLC421 ($guaA^-guaB^-$) (J. Bact., 139 (1979):320, E. coli K294 (r^-, m^+) (FERM BP-526), B. coli K294/pXA10 (FERM-BP 399), and the like can be exemplified.

The manufacture of a plasmid (pXA10), in which a DNA fragment containing a guaA gene of an E. coli chromosome is inserted into the tetracycline-resistant position of a vector, plasmid pBR322, and a tryptophan promoter (Ptrp) is linked to this plasmid upstream from the guaA gene, and an E. coli strain, K294/pXA10, obtained by transforming

the K294 strain with pXA10 is shown in Reference Example 1.

By culturing such E. coli in the usual culturing method, a culturing fluid, bacterial cells, or their treated products having powerful activity for producing the GMP from the XMP, ammonia and/or L-glutamine, and an energy donor other than a phosphorus oxide can be obtained. That is, such E. coli should be cultured by adjusting the temperature, pH, and the like under aerobic conditions in the usual medium containing a carbon source, nitrogen source, inorganic matter, amino acids, vitamins, and the like.

A hydrocarbon, such as glucose, lactose, sucrose, maltose, mannitol or sorbitol; sugar alcohol, glycerol, starch hydrolysis liquid, molasses, and the like may be used for the carbon source. Moreover, various organic acids, such as pyruvic acid, lactic acid and citric acid, and further, various organic acids, such as glutamic acid and methionine, also can be used.

Various inorganic and organic ammonium salts, such as ammonia or ammonium chloride, ammonium sulfate, ammonium carbonate and ammonium acetate; amino acids, such as glutamic acid, glutamine, and methionine; various sources, such as nitrogen-containing organic matter, like peptone, NZ-amine, corn steep liquor, meat extracts, yeast extracts, casein hydrolysates, fish meal, or digested products and hydrolysates thereof, and the like, can be used for the nitrogen source.

Potassium dihydrogen phosphate, sodium monohydrogen phosphate, magnesium sulfate, sodium chloride, calcium chloride, iron chloride, copper sulfate, manganese chloride, ammonium molybdenate, zinc sulfate, and the

like may be added, as needed for the inorganic matter. Components other than the vitamins, amino acids and nucleic acids required for growing microorganisms thus supplied in the medium due to the other culture components as mentioned above, need not be added in particular.

Culturing is performed under aerobic conditions, such as a shaking culturing or aeration stirring culturing. The culturing temperature should be 20 to 50°C, and preferably, 28 to 42°C. It is desirable to keep the pH of the medium as neutral as possible during culturing. The culturing time is normally 1 to 24 hours.

A cultured product of E. coli obtained by the above process is catalyzed, as is or as a treated product obtained by treating this cultured product in various ways, with XMP, ammonia and/or L-glutamine, and an energy donor other than a phosphorus oxide. A condensate of the cultured product, a supernatant obtained by centrifuging a dried product of the bacterial cells, the dried product of the bacterial cells, an acetone-treated /549 product, a surfactant- and/or organic solvent-treated product, a bacteriolysis enzyme-treated product, solidified bacterial cells or an extracted enzyme authentic preparation from bacterial cell bodies, and the like can be cited for the treated product.

Any catalytic reaction can be performed as long as it is in a water-based medium, and most ideally, the GMP is accumulated in a culturing liquid, with XMP, ammonia and/or L-glutamine, and an energy donor other than a phosphorus oxide, and as further needed, phosphate ions, magnesium ions, and further, a surfactant and/or organic solvent present in the microorganism culturing liquid, or a culturing liquid, bacterial cell suspension, XMP,

ammonia and/or L-glutamine and energy donor other than a phosphorus oxide may be added to a treated product thereof, and the GMP can be accumulated by reacting this for 1 to 48 hours at 20 to 50°C. In this case, it is desirable to adjust the pH to 6 to 10, and more preferably, 7 to 8. The concentration (g/L) of each matrix in each medium or reaction liquor is 1 to 100 XMP, 25 or less $(NH_4)_2SO_4$, and 25 or less L-glutamine.

Besides highly purified products, any source containing XMP can be used for the XMP source as long as the accumulepation of GMP is not hindered, such as an XMP fermentation liquor of a microorganism, a condensate thereof, and further, a partially purified authentic preparation thereof.

As long as it is an unphosphorylated compound, or an energy donor that can used by the E. coli in use, a hydrocarbon, such as glucose, arabinose, lactose, maltose, sucrose, mannitol, sorbitol, trehalose, molasses, or a starch hydrolysate; an organic acid, such as pyruvic acid, lactic acid, acetic acid or α-ketoglutaric acid; an amino acid, such as glycine, aspartic acid or glutamic acid; and the like can be used for the energy donor which is used in a concentration of 1 to 200 g/L.

It is desirable to keep the concentration of the phosphate ions and magnesium ions in the catalytic reaction within a range of 4 to 400 mM. It is not necessary to add ions when the amount brought from the culturing liquid or bacterial cells into the reaction solution satisfies this concentration range. But on the other hand, they may be added so that this concentration is adjusted to within the above-mentioned concentration range in unsatisfactory cases. A sodium salt, potassium salt, magnesium salt, or the like of phosphoric acid can be used for the phosphate ions.

Moreover, either an inorganic salt or salt of an organic acid can be used for the magnesium ions.

A cationic surfactant, such as polyoxyethylene stearylamine (e.g., Nymine S-215, made by NOF Corp.), or cetyltrimethyl ammonium bromide; an anionic surfactant, such as sodium oleylamidesufluric acid; an amphoteric surfactant, such as polyoxyethylene sorbitan monostearate (e.g., Nonion ST221, made by NOF Corp.), and the like can be used for the surfactant. These surfactants be used normally at a concentration of 0.1 to 50 mL/L, and preferably, 1 to 20 mL/L.

The usual method in which activated charcoal, an ion-exchange resin, and the like is used can be used for the method for collecting the GMP accumulated in the water-based reaction liquor.

The practical examples of the present invention will now be shown.

Practical Example 1

A 1 L Erlenmeyer flask containing 200 mL of an MP medium (6 g/L disodium phosphate, 3 g/L potassium phosphate, 5 g/L sodium chloride, 1 g/L ammonium chloride, 4 mg/L thiamine hydrochloride, 250 mg/L magnesium sulfate, and 3 g/L glucose) was inoculated with E. coli K294 (FERM BP-526) strain, and an overnight reciprocal shaking culturing was performed at 28°C. The bacterial cells were gathered by centrifuging and preserved by being frozen at -20°C.

Each component was dissolved in a liquid in which the frozen bacterial cells were hydrolyzed at room temperature so that the final concentration thereof was 200 g/L and the concentration of the XMP-$Na_2 \cdot 7H_2O$ was 40 g/L, the concentration of glucose was 50 g/L, the concentration of the sodium

phylate was 2 g/L, the concentration of the Na_2HPO_4 was 5 g/L, and the concentration of the $MgSO_4 \cdot 7H_2O$ was 5 g/L. Each of these was fractionated 20 mL each into a 200 mL beaker. Each fraction was maintained at 37°C in a constant temperature water bath, which was stirred at 900 rpm with a magnetic stirrer, and XMP was converted to GMP after storing each fraction for 24 hours while adjusting the pH to 7.4 with 9% aqueous ammonia (see Table 1). The reaction liquor having the above composition was used as is in (1) in Table 1, and also a reaction liquor in which 4 g/L of Nymine (abbreviated "NIM," hereafter) S-215 and 10 mL/L of xylene were added to the above composition in (2) was used.

Table 1

NIM S-215 and Xylene	$GMP \cdot Na_2 \cdot 7H_2O$ (g/L)
(1) -	2.2
(2) +	5.2

Practical Example 2 /550

An E. coli K294 strain and E. coli K294/pXA10 (see body of text) having a plasmid containing a guanylic acid synthase gene were cultured as in Practical Example 1 (providing an M9 medium in which 50 mg/L of ampicillin were added to a culture of K294/pXA10 was used). As a result of a reacting a liquor containing NIM S215 and xylene at the same conditions in Practical Example 1-(2), 5.5 g/L of $GMP \cdot Na2 \cdot 7H_2O$ were produced in 23 hours with the E. coli K294 strain and 23.8 g/L of $GMP \cdot Na2 \cdot 7H_2O$ were produced in 6 hours with the E. coli K294/pXA10 strain.

Practical Example 3

The E. coli K294/pXA10 strain was cultured as in Practical Example 1 in an M9 medium containing 50 mg/L of ampicillin. The amount of bacterial

cells was 7.5 g/L by weight as wet bacterial cells. XMP, glucose, sodium phylate, Na_2HPO_4, $MgSO_4 \cdot 7H_2O$, NIM S-215, and xylene were added to this culturing liquid so that each concentration thereof was 40 g/L, 50 g/L, 2 g/L, 5 g/L, 5 g/L, 4 g/L and 10 mL/L, respectively. As a result of performing transformation from XMP to GMP as in Practical Example 1, 5.2 g/L of $GMP \cdot Na_2 \cdot 7H_2O$ were produced in 23 hours.

Reference Example 1

Creation of recombinant plasmid expressing GMP synthase efficiently: 1) An L medium containing 10 g/L of Bactotrypton (made by Difco), 5 g/L of a yeast extract (made by Difco), and 5 g/L of sodium chloride and whose pH was adjusted to 7.2 was inoculated for 18 hours at 30°C with an E. coli JA200 strain possessing gua operon derived from E. coli chromosome (including guaA and guaB) and a ColE1 hybrid plasma, pLC 34-10. This plasmid, pLC 34-10, was separated and purified in accordance with a known method (Nucleic Acids Research 7 (1979):1513) from the resulting cultured bacterial cells. pBR322 used as the vector also was separated and purified from an E. coli JA 194 strain (B. Ratzkin & J. Carbon, Proc. Natl. Acad. Sci., U.S.A 74 (1977):487), which is a stock strain thereof, in the same method. Moreover, as long as not expressedly stated, the L medium was used to culture and preserve the bacteria.

The pLC 34-10 was about 15 kilobases (abbreviated "Kb," hereafter) in size, which was cleaved at one 1 site by a restriction enzyme, EcoRI, and at three 3 sites by PstI (see Figure 1). A plasmid fragment having a PstI cleavage site (about 3.6 Kb) was purified.

A DNA fragment derived from about 0.2 μg of the pLC 34-10 obtained by the above process and a DNA fragment derived from about 0.05 μg of the pBR322 were treated for 18 hours at 4°C with 20 mM

Tris-hydrochloric acid (pH: 7.6), 2 units of T4 ligase in 40 μL of a buffer containing 10 mM $MgCl_2$, 10 mM dithiothreitol and 0.5 mM ATP (called "T4DNA ligase buffer," hereafter). The recombinant plasmid DNA obtained by the above process was used to transform an E. coli PL1068 strain with a variation of guaA in a method by Cohen, et al. (Proc. Natl. Acad. Sci., USA. 69 (1972):2100, to obtain a transformed strain resistant to tetracycline (20 μg/mL) and in which the guanine requisite by the host is lost.

As a result of analyzing the structure of the plasmid by isolating and purifying the plasmid from this transformed strain and digesting the DNA with a restriction enzyme, such as EcoRi and PstI, it was confirmed that it was a recombinant plasmid (named pXA1) in which about 7 Kb of EcoRI-PstIDNA fragment derived from the pLC34-10 was inserted into the EcoRi-PstI site of the pBR322. Upon covering the E. coli KLC421 ($guaA^-$, $guaB^-$) transformed by using this pXA1 on an MP plate medium (a medium containing NH_4Cl (1 g), Na_2HPO_4 (6 g), KH_2PO_4 (3 g), NaCl (5g), $MgSO_4 \cdot 7H_2O$ (0.25 g), glucose (3 g), vitamin B_1 (4 g) and casamino acid (2 g) in 1L of water, with 1.5% agar added) and investigating growth, the E. coli grew on a plate culture containing 5 mg/L of xanthine or guanine but did not grow on an M9 plate containing 5 mg/L of hypoxanthine. This result shows that, of the guaA and guaB genes derived from pLC34-10, only the guaA was present in the pXA1. The E. coli K294 was transformed by using pXA1 and the resulting bacterial strain was deposited as E. coli K294/pXA1

(FERM BB-498) at the Fermentation Research Institute on March 8, 1984.

2) Linkage of a trp promoter (abbreviated "Ptrp," hereafter) upstream from guaA:

3 µg pXA1 DNA were dissolved in 30 µL of a Y-50 buffer, 15 units of HindIII were added, a digestion reaction was performed for 2 hours at 37°C, 2 µL of 2M NaCL and 15 units of MIuI were added thereto, and a 2-hour digestion reaction was performed at 37°C. A smaller DNA fragment (about 3.3 Kb) containing guaA heat-treated for 10 minutes at 65°C was purified. Meanwhile, pGBK2 was used for the plasmid having Ptrp. /551 Moreover, a method for creating pGBK3 is shown in References Examples 2 and 3. There is a HindIII cleavage site downstream from the Ptrp of the pGBK3 plasmid (see Figure 1).

3 µg pGBK3 DNA were digested with HindIII and M1uI in the same method as mentioned above, and a larger DNA fragment (about 4 Kb) containing Ptrp was purified. 1 unit of T4DNA ligase was added to a 20 µL T4DNA ligase suspension and an 18-hour fixation reaction was performed at 4°C on about 0.2 µg of a DNA fragment derived from the resulting pXA1 and about 0.1 µg of a DNA fragment derived from pGBK3. A transformed strain resistant to ampicillin was obtained by using the recombinant plasmid obtained by the above process to transform the E. coli K294 strain. The plasmid DNA was isolated and purified from this transformed strain and upon analyzing the structure thereof, it was confirmed that it had a structure in which a DNA fragment containing pXA1-derived guaA was inserted downstream from the pGBK3-derived Ptrp, and this plasmid was named pXA10 (see Figure 1). The E. coli strain possessing pXA10 was deposited at the Fermentation

Research Institute on March 8, 1984 as E. coli K294/pXA10 (FERM BP-499).

3) GMP synthase activity of recombinant plasmid stock strain:

The measurement of the GMP synthase activity was carried out by slightly modifying a known method (J. Biol. Chem. 226 (1957):351-363), as mentioned below.

An M9 liquid medium was inoculated with an E. coli seed culture offered in the activity measurement test, subjected to a shaking culturing for 18 hours at 30°C, the culturing liquid was subsequently diluted with distilled water or suspended in a suitable amount of distilled water after centrifuging to concentrate it, toluene was added thereto so that the final concentration was 20 mL/L, and this was shaken for 20 minutes at 37°C.

A transformation reaction from XMP to GMP was performed by allowing this toluene-treated culturing liquid to coexist in a reaction liquor having a composition comprising 160 mM Tris-hydrochloric acid (pH: 8.6), 12mM ATP, 25 mM XMP, 16 mM $MgSO_4 \cdot 7H_2O$, and 40 mM $(NH_4)_2SO_4$, and shaking it at 42°C.

The production of GMP was quantified by measuring the absorbance of the supernatant at 290 nm after collecting the reaction liquor over time, mixing it with 40 times the volume of 3.5% perchloric acid, and centrifuging it.

A GMP synthase activity of the bacterial cells used or obtained in the present invention is shown in Table 2.

The activity is defined as the amount of activity for producing 1 μmol of GMP per minute (1 unit).

Table 2

Host E. coli strain	Plasmid	Deposit No.	Activity per Wet Bacterial Cells (unit/g-wet bacterial cells
K294	-	FERM BP-526	0.88
K294	pXA1	FERM BP-498	17.08
K294	pXA10	FERM BP-499	16.91

Reference Example 2

Creation of recombinant plasmid, pGKA2, expressing human interferon (IFN) (see Figures 2 and 3):

6 μ g of a plasmid, pIFNγ-G4 (collected from ATCC39123 in the aforementioned plasmid isolation method) were dissolved in a total 50 μL solution containing 20mm Tris-hydrochloric acid (Tris-HCl) (pH: 7.5), 10 mM $MgCl_2$, 10 mM dithiothreitol and 50 mM NaCl, 12 units of a restriction enzyme, PvuII and 12 units of HindIII were added, and a 4-hour digestion reaction was performed at 37°C. The enzymes were deactivated by heat treating the reaction liquor at 65°C for 7 minutes, and purified in a low-melting point agarose gel electrophoresis method to obtain 1.2 μg of a DNA fragment containing a 1.3 Kb human IFN-γDNA.

Separately 4 μg of pKYP11 were dissolved in a 40 μL total solution containing 10 mM $MgCl_2$, 10 mM dithiothreitol and 50 mM NaCl, 3 units of BamHI were added, and a 3-hour digestion reaction was performed at 37°C. The enzyme was deactivated by heat treating the reaction liquor at 65°C for 5 minutes. 30 μM each of dATP, dCTP, dGTP and dTTP were added, 8 units of an E. coli DNA polymerase I (Klenow fragment, made by New England Biolabs, 1 μL), and a fill-in reaction was performed at 15°C for 1 hour. In order to deactivate the DNA polymerase I, a 15-minute heat treatment at 68°C was performed, after which 10 units of HindIII were added, and

a digestion reaction was further performed at 37°C for 3 hours, after which the HindIII was heat-treated again for 5 minutes at 654°C to deactivate it.

About 2.5 μ g of an approximately 4.7 Kb DNA fragment containing Ptrp was obtained from the digested reaction liquor of the plasmid pKYP11 obtained by the above process after purifying it in a low-melting /552 point agarose gel electrophoresis method.

0.5 μg of the DNA fragment (1.3 Kb) containing human IFN-γ DNA and about 1.0 μg of a DNA fragment (4.7 Kb) containing Ptrp obtained from a plasmid, pKYP11, were dissolved in 20 μL containing 20 mM Tris-HCl (pH: 7.5), 6 mM $MgCl_2$, 5 mM dithiothreitol and 500 μM ATP, 4 units of T4DNA ligase (made by New England Biolabs) were added thereto, and a fixation reaction was performed for 18 hours at 4°C. An E. coli HB101 strain was transformed as in the usual method using the resulting recombinant plasmid mixture to obtain an Ap^R colony. The plasmid was isolated from the culturing liquid of this colony to obtain the pGC7 shown in Figure 2. The pGC7 structure was confirmed after digestion with HindIII, BamHI, HpaI, SalI, BcoRI and ClaI in an agarose gel electrophoresis method. The E. coli strain containing pGC7 was deposited at the Fermentation Research Institute as Escherichia coli IGC7 (FERM P-6814, and FERM BP497).

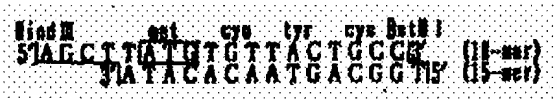
(b) Creation of recombinant plasmid pGKA2:

6 μg of the pGC7DNA obtained in Reference Example 2(a) were dissolved in a total 50 μL solution containing 20 mM Tris-HCl (pH: 7.5), 10 mM MgCl, 10 mM dithiothreitol and 10 mM NaCl, 12 units of a restriction enzyme, BstNI (made by New England Biolabs) were added, reacted for 3 hours at

60°C, after which NaCl was added to get 150 mM, 18 units of SaL were added, and a 3-hour digestion reaction was further performed at 37°C. The SalI was deactivated under heating for 5 minutes at 65°C again, and purified in a low-melting point agarose gel electrophoresis method to obtain 0.8 μg of an approximately 1,125 bp DNA fragment containing a majority of human IFN-γDNA.

3 μg of pKYP10 were dissolved in a 40 μL total solution containing 20 mM Tris-HCl (pH: 7.5), 10 mM $MgCl_2$, 10 mM dithiothreitol and 100 mM NaCl, 6 units each of the restriction enzymes, HindIII and SalI, were added thereto, and a digestion reaction was performed for 3 hours at 37°C. This was heated for 5 minutes at 65°C to deactivate the HindIII and SalI. This digested reaction liquor was purified in a low-melting point agarose gel electrophoresis method to obtain about 1.8 μg of an approximately 4.1 Kb DNA fragment.

Meanwhile, since the N-terminal of the mature human IFN-γ polypeptide was Cys, in order to express the mature IFN-γDNA, the following DNA linker was synthesized from the reasoning that it was necessary to provide an initiation codon (ATG) just before the 5'terminal TGT (Cys) and a suitable length was required for the distance between SD-sequence downstream from the Ptrp and ATG.



First of all, single-strand 18-mer and 15-mer DNA were synthesized in the usual triester method (R. Crea, et al. Proc. Natl. Acad. Sci. 75 (1978):5765). 2 μg each of the 18-mer and 15-mer were dissolved in a 20 μL total solution containing 50 mM Tris-HCl (pH: 7.5), 10mM $MgCl_2$,

5 mM dithiothreitol, 0.1 mM EDTA and 1 mM ATP, 30 units of T4 polynucleotide kinase (made by Boehringer Mannheim) were added thereto, and a 60-minute phosphorylation reaction was performed at 37°C.

0.4 μg of the pGC7-derived BstNI-SalI fragment (1,125 bp) obtained above and the expression vector pKYP10 were digested with HindIII and SalI, 1.0 μg of the resulting DNA fragment (4.1 Kb) was dissolved in a 25 μL total solution containing 20 mM Tris-HCl (pH: 7.5), 6 mM $MgCl_2$, 5 mM dithiothreitol and 500μm ATP, and about 0.1 μg of the above-mentioned DNA linker was added to this mixture. 6 units of T4DNA ligase were further added to this mixture, and a 17-hour fixation reaction was performed at 4°C. An E. coli HB101 strain was transformed in the usual method using the resulting recombinant plasmid mixture to obtain an Ap^R colony. The plasmid was isolated from this colony culture to obtain the pGKA2 shown in Figure 3. The structure of the pGKA2 was confirmed in an agarose gel electrophoresis method after digestion with EcoRI, ClaI, HindIII, BstNI and SalI. It was confirmed that the base sequence between the SD-sequence (AAGG) of the plasmid, pGKA, and the initiation codon (ATG) was AAGGGTATCGATAAGCTTATG in a Maxam Gilbert method (A.M. Maxam, et al.: Proc. Natl. Acad. Sci., 74 (1977):560).

The fact that the human IFN-γDNA of the pGKA2 had an RsaI unit differs from what is known in that the human IFN-γ polypeptide that this DNA codes is the 9th amino acid glutamine (Gln).

Furthermore, the synthetic DNA used above is different from the /553 underlined parts of  , which P.W. Gray, et al. used. In this way, in the pGKA2, the restriction enzyme unit present in the

BastNI which is (CCAGG) in the DNA region coding the human IFN-γ, and the pGKA2 differs from the known one thereby. Moreover, the distance between and the structure of the SD-sequence and the ATG are important because they significantly affect expression of the protein by E. coli. But the base sequences between the SD-sequence of the pGKA2 and the ATG clearly differ from those of known recombinant plasmid, pIFN-γtrp48 (P.W. Gray, et al.)

E. coli containing pGKA2 is deposited at the Fermentation Research Institute as Escherichia coli IGKA2 (FERM P-6798, and FERM BP-496.

Reference Example 3

Creation of recombinant plasmid pGBK3 expressing human IFN-γ under control of tacI promoter:

First of all, the posttransciptional site of the E. coli lipoprotein (lpp) gene of the IFN-γ-epxressing plasmid pGKA2 (see Reference example 2 in creation of pKAG2) (abbreviated "lpp terminator," hereafter) was introduced in accordance with the procedure in (a), (b) and (c) below (see Figure 4).

(a) Creation of pBGDI:

2 μg of about a 3.6 Kb of plasmid, pIFNγ-G4, were dissolved in 20 μL of a Y-50 buffer, 6 units of pvuII were added, digestion was performed for 2 hours at 37°C, and the reaction was subsequently stopped by a heat treatment for 10 minutes at 65°C. 0.1 μg of this digested product was subjected to a fixation reaction for 18 hours at 4°C by 2 units of T4DNA ligase in 20 μL of T4DNA ligase buffer in the presence of 5'-phosphorylted BamHI linker-(5'-pCCGGATCCGG-3'; made by Collaborative Research).

An Ap-resistant colony was obtained by using the recombinant plasmid DNA obtained by the above process and transforming the E. coli HB101 strain. As a result of analyzing the structure of the plasmid by isolating the plasmid DNA from this transformed strain and digesting this DNA with BamHI or the like, it was confirmed that a recombinant plasmid, pGBD1, in which a BamHI linker was inserted into the PvuII site of pIFNγ-G4 was obtained.

(b) Creation of pKYP14:

The creation of the recombinant plasmid, PKYP14, used as the source for supplying 1pp terminator is mentioned next.

5 μg of a plasmid, pKYP10, which transports the trp promoter (Tokkai No. 58-110600), were dissolved in 40 μL of Y-100 buffer, 10 units of BamHI were added, and a digestion reaction was performed for 2 hours at 37°C. Then, 1 μL of Y-100 buffer, 2.5 μL of 1M NaCl, 5.5 μL distilled water and 20 units of SalI were added thereto, and a reaction was further performed for 2 hours at 37°C. After a 10-minute heat treatment at 65°C, a larger plasmid DNA fragment (about 4.9 Kb) was purified using a low-melting point agarose electrophoresis method. Meanwhile, 5 μ g of a plasmid, pIN-II-A1 transporting a 1pp terminator (K. Nakamura, et al.: The EMBO Journal 1 (1982):771; (same as pKEN045 in the publication of Tokkai No. 57-140800) was digested by BamH1 and SalI as above. An approximately 0.95 Kb BamHI-SalI fragment containing the resulting 1pp terminator was purified.

About 0.1 μg of a pKYP10-derived DNA fragment obtained by the above process and about 0.05 μg of a pIN-II-A1-derived DNA fragment were subjected to a fixation reaction for 18 hours at 4°C by adding 1 unit of T4DNA ligase to 20 μL of a T4DNA ligase buffer.

Upon isolating the plasmid DNA from the transformed E. coli HB101 using the recombinant plasmid DNA obtained by the above process, it was confirmed that the lpp terminator was inserted downstream from the plasmid pKYP14 of the IKYP14 strain.

(c) Creation of pGBJ2:

The lpp terminator was introduced as follows downstream from the IFN-γDNA by recombining the recombinant plasmid, pGBD1 and the pKYP14 obtained in (a) and (b) above.

5 µg (about 3.6 Kb) of the plasmid, pGMDI, were dissolved in 30 µL of a buffer comprising 10mM Tris-HCl (pH: 7.5), 7 mM $MgCl_2$, and 6 mM 2-mercaptoethanol (abbreviated "Y-0 buffer," hereafter), 10 units of ClaI were added, and a 2-hour digestion reaction was performed at 37°C. After it was cooled in ice after a 10-minute heat treatment, 2 µL of a 10-fold concentrated Y-0 buffer, 5 µL of 1M NaCl, 12 µL of distilled water and 2.0 units of the restriction enzyme BamHI were added and mixed after which a 2-hour digestion reaction was performed at 37°C. Due to this /554 reaction, the plasmid DNA was digested partially by the BamHI. The resulting ClAI-BamHI DNA fragment (about 1.3 Kb) containing the IFN-γDNA was purified. Meanwhile, 5 µg of a plasmid, pKYP14 (about 5.8 Kb) containing the lpp terminator were digested for 2 hours with 10 units of GlaI and 20 units of BamHI in 50 µL of the Y-50 buffer, after which an approximately larger plasmid DNA fragment (5.0 Kb) containing lpp terminator was performed. The pKYP14-derived DNA fragment (about 0.1 µg) and the pGBD1-derived DNA fragment (about 0.05 µg) obtained by the above process were subjected to a fixation reaction for 18 hours at 4°C in 20 µL of a T4DNA ligase

buffer after adding 1 unit of T4 DNA ligase.

The plasmid DNA was isolated from the E. coli HB101 strain transformed using the recombinant plasmid obtained by the above process, and upon analyzing its structure, it was confirmed that the plasmid, pGBJ2 of the IGBJ2 strain had a structure in which the lpp terminator was introduced downstream from the IFN-γDNA.

(d) Creation of pGBK3:

The plasmid, pGBJ2, and IFN-gamma-expressing plasmid, pGKA2 (see Reference Example 2) obtained in (c) above were recombined and a plasmid pGBK3 having a structure in which the lpp terminator was introduced downstream from the IFN-γDNA was created as follows.

About 5 μg of the plasmid, pGKA2 (about 5.2 Kb), were dissolved in 30 μL of a Y-50 buffer, 10 units or more of PstI were added, and a 2-hour digestion reaction was performed at 37°C. After cooling it in ice after a 10-minute heat treatment at 65°C, 2 μL of a 10-fold concentrated Y-150 buffer, 3 μL of 1M NaCl, 14 μL of distilled water, and 10 units of a restriction enzyme, NcoI (made by New England Biolabs; the same hereafter) were added, and a 2-hour digestion reaction was performed at 37°C. After a 10-minute heat treatment at 65°C, an approximately 1.85 Kb plasmid DNA fragment containing IFN-γDNA was purified. Next, the same treatment as the treatment above was conducted on about 5 μg of the recombinant plasmid, pGBJ2 (about 6.4 Kb), obtained above in which it was added to pGKA2, and an approximately 4.7 Kb PstI-NcoI plasmid DNA fragment was purified. About 0.1 μg of the pGKA2-derived DNA fragment and about 0.1 μg of the pGBJ2-derived DNA fragment obtained in the above process were subjected to a 10 hour fixation reaction

at 4°C in 20 μL of a T4DNA ligase buffer after adding 1 unit of T4DNA ligase. Plasmid DNA was isolated from the E. coli HB101 strain transformed using the recombinant plasmid obtained by the above process, and isolated, and upon analyzing its structure, it was confirmed that the plasmid, pGBK3, of the IGBK3 strain had the target structure.

4. Brief Description of the Drawings

Figure 1 shows the creation step of the plasmid, pXA10.

Figure 2 shows the creation step of the plasmid, pGC7.

Figure 3 shows the creation step of the plasmid, pGKA2.

Figure 4 shows the creation step of the plasmid, pGBK3.

Figure 1